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Utilization of Non-Sugar Sources for Vitamin B_{12} Production

TADASHI KAMIKUBO,[1] MITSUNORI HAYASHI,[2] NAOMICHI NISHIO,[2]* AND SHIRO NAGAI[2]

Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Sakyo, Kyoto,[1] and Department of Fermentation Technology, Faculty of Engineering, Hiroshima University, Sendamachi, Hiroshima,[2] Japan

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Assimilation of non-sugar carbon sources for vitamin B_{12} production was studied.

It is well known that some microorganisms, especially bacteria of the genera *Propionibacterium* (P. G. Lim, Chem. Abstr. **70**:210, 1969; U.S. Patent 3411991, 1968) and *Streptomyces* (3) produce 40 mg or more of vitamin B_{12} per liter (P. Rapp, Ph.D. thesis, University of Stuttgart, Stuttgart, Germany), when grown on media containing sugars as carbon sources. Only a few reports have been published on bacterial production of vitamin B_{12} from non-sugar carbon sources (1, 2, 4–6).

In the present study, *Pseudomonas aureofaciens* Institute for Fermentation, Osaka, Japan (IFO) 3521, *P. ovalis* IFO 3738, *P. aeruginosa* IFO 3080, *Mycobacterium smegmatis* IFO 3803, *Nocardia gardneri* IFO 3385, *Rhodopseudomonas sphaeroides,* and *Bacillus badius* were used for the study of vitamin B_{12} production from hydrocarbons. *Klebsiella* sp. 101, *Pseudomonas* sp. ATCC 14718, and *Microcyclus eburneus* ATCC 21373 were employed for studies on B_{12} formation using methanol as substrate. The hydrocarbon-assimilating bacteria were grown in a medium containing 1.32 g of $(NH_4)_2SO_4$, 0.6 g of $(NH_2)_2CO$, 2.0 g of KH_2PO_4, 3.0 g of $Na_2HPO_4 \cdot 12H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of Na_2CO_3, 10 mg of $CaCl_2 \cdot 2H_2O$, 5 mg of $FeSO_4 \cdot 7H_2O$, 2 mg of $MnSO_4 \cdot nH_2O$, and 1 mg of $CoSO_4 \cdot 7H_2O$ per liter of the medium. An appropriate *n*-paraffin was added in the amount of 1% (vol/vol). The methanol-assimilating bacteria were cultivated usually in a medium containing 1.5 g each of $(NH_4)_2SO_4$, $(NH_2)_2CO$, NH_4NO_3, KH_2PO_4, and K_2HPO_4; 0.3 g of $MgSO_4 \cdot 7H_2O$; 0.1 g of yeast extract; 10 mg each of $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, and $ZnSO_4 \cdot 7H_2O$; 1 mg of $MnSO_4 \cdot nH_2O$; 2 mg of $CoCl_2 \cdot 6H_2O$; 1 mg of thiamine-hydrochloride; and 10 ng of biotin per liter of the medium (pH 7.0). Methanol, usually 2% (vol/vol), was added to the medium as sole carbon source. Batch cultures of *Klebsiella* sp. 101 and *Pseudomonas* sp. ATCC 14718 were carried out in a 30-liter Marubishi jar fermentor (type MSJ, working volume = 20 liters). The dissolved oxygen in the culture was maintained during the cultivation above 4 to 5 mg/liter with *Pseudomonas* sp. ATCC 14718 and about 3 mg/liter with *Klebsiella* by varying the agitation speed and aeration rate. The batch feeding was devised so that methanol required for the growth could be supplied at the predetermined level by adjustment of pH with the methanol-ammonia solution. Batch-feeding was started when the residual methanol concentration in a batch culture fell to about 0.5% (vol/vol) from the initial value of 1%, and cultivation was continued with intermittent addition of the methanol-ammonia solution in response to pH decrease. From several experiments, the ratios of methanol (99% purity) to ammonia (29% wt/vol) required in the mixture to compensate for the methanol consumed and simultaneously to adjust pH were determined to be 100:23 (vol/vol) for *Pseudomonas* sp. ATCC 14718 and 100:18.3 (vol/vol) for *Klebsiella* sp. 101.

Production of vitamin B_{12} in a medium containing a pure *n*-paraffin or *n*-paraffin mixtures as the carbon source by seven bacteria was examined. Table 1 shows the highest amount of vitamin B_{12} produced from the respective pure hydrocarbon; the amount obtained was less than 100 μg/liter. *P. aeruginosa* and *R. sphaeroides* produced more intracellular vitamin B_{12} in the media containing *n*-paraffin mixture than in pure *n*-paraffin media, and the total B_{12} potency ranged from 150 to 200 μg/liter. On the other hand, the major part of the vitamin B_{12} produced by *B. badius* was found in the extracellular fluid.

Vitamin B_{12} production by three bacteria in a medium containing 2% (vol/vol) of methanol is given in Table 2. The potencies ranged from 150 to 260 μg/liter, and the vitamin was present in the cells mainly as adenosylcobalamin and methylcobalamin. These three methanol-assimilating microorganisms utilized carbon sources other than methanol and produced vitamin B_{12} in different amounts, depending on the carbon source. Glucose, ethanol, tricarboxylic acid cycle intermediates, 1,2-propanediol, glycerol, and methanol were tested for their ability to support cellular growth and vitamin B_{12} production. As shown in Table 2, these organisms utilized methanol, ethanol, glucose, glycerol, and 1,2-propanediol. Vitamin B_{12} was produced when methanol was used as a sole carbon source for *Klebsiella* and when methanol or propanediol was used for *Pseudomonas* ATCC 14718. *Microcyclus* produced larger amounts of vitamin B_{12} on all substrates tested.

To increase the production of vitamin B_{12}, we have attempted batch feeding using a 30-liter fermentor for *Klebsiella* sp. 101 and *Pseudomonas* sp. ATCC 14718. Figure 1 shows the time course of microbial growth as well as vitamin B_{12} production. In *Klebsiella,* 1.3 mg of vitamin B_{12} per liter and 18.3 g (dry cell weight)/liter were

TABLE 1. *Hydrocarbon assimilation and vitamin B_{12} production by bacteria*

Organism	Dry cells (g/liter)	Vitamin B_{12} (μg/liter) Intracellular	Extracellular	Hydrocarbon
Pseudomonas aureofaciens	2.0	15.5	21.0	*n*-Dodecane
P. ovalis	3.1	36.2	40.0	*n*-Decane
P. aeruginosa	3.6	28.0	40.0	*n*-Octadecane
	8.1	153	2.5	P-4[a]
Mycobacterium smegmatis	4.6	25.0	3.8	*n*-Decane
Nocardia gardneri	2.6	43.0	21.0	*n*-Decane
Rhodopseudomonas spheroides	12.0	212	5.2	P-4[a]
Bacillus badius	3.9	4.2	140	SHP[b]

[a] P-4 contained mainly *n*-tridecane (40 to 50%) and *n*-tetradecane (30 to 42%).

[b] SHP (Super Heavy *n*-Paraffin Mixture) contained mainly *n*-pentadecane (72%) and *n*-hexadecane (19%).

TABLE 2. *Utilization of various carbon sources and vitamin B_{12} production by methanol-utilizing bacteria*

Bacterium	Carbon source	Initial concn[a]	Growth (OD_{570})[b]	Vitamin B_{12} (μg/liter)
Klebsiella sp. 101	Glucose	2	6.0	2.5
	Ethanol	2	5.6	20.0
	Succinic acid	2	4.6	34.7
	Malic acid	2	3.0	52.7
	Methanol	0.5	4.7	49.6
		2	10.7	156
	1,2-Propanediol	2	12.5	55.0
M. eburneus ATCC 21373	Glucose	2	12.3	80.0
	Ethanol	1	5.2	81.0
	Acetic acid	1	1.6	33.2
	Glycerol	1	7.0	70.0
		2	17.2	102
	Methanol	1	4.2	57.0
		2	7.0	102
	1,2-Propanediol	1	6.2	69.8
		2	13.5	110
Pseudomonas sp. ATCC 14718	Glucose	1	2.5	8.0
		2	3.0	10.0
	Ethanol	1	2.5	53.0
	Glycerol	1	2.9	16.0
		2	2.6	15.0
	Methanol	1	4.1	101
		2	4.8	156
	1,2-Propanediol	2	7.5	118

[a] Percentage (wt/vol) for glucose, succinic acid, malic acid, and glycerol, and percent (vol/vol) for ethanol, methanol, acetic acid and 1,2-propanediol. Cultivation was carried out on a reciprocal shaker (115 rpm) for 3 to 7 days at 28°C in Sakaguchi flasks containing 100 ml of medium.

[b] OD_{570}, Optical density at 570 nm.

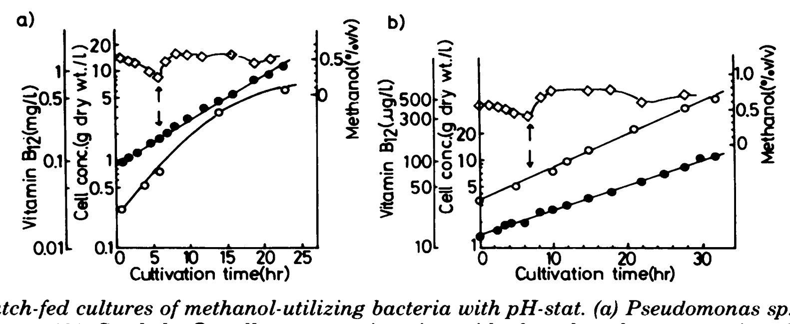

FIG. 1. *Batch-fed cultures of methanol-utilizing bacteria with pH-stat. (a) Pseudomonas sp. ATCC 14718, (b) Klebsiella sp. 101. Symbols: ●, cell concentration; ◇, residual methanol concentration; ○, vitamin B_{12} concentration; ↕ start of the batch-feeding with pH-stat. Final biomass and vitamin B_{12} concentrations were 18.3 g (dry weight)/liter and 1.3 mg of B_{12} per liter after 55.5 h of cultivation in Klebsiella, and 35.7 g (dry weight)/liter and 3.2 mg of B_{12} per liter after 85 h of cultivation in Pseudomonas.*

produced after 55 h of cultivation, whereas in *Pseudomonas,* 3.2 mg of the vitamin per liter and 35.7 g (dry cell weight)/liter were formed by 85 h of cultivation. These data represent a higher yield of vitamin B_{12} (corresponding to 8- and 12-fold, respectively) than that in the batch cultures.

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